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(54) DNA denaturation by an electric potential

(57) A process is described for denaturing DNA into its individual strands in an electrochemical cell in the presence of a mediator compound that transfers charge from an electrode to the DNA in solution. The process may be used in the replication of DNA by a polymerase chain reaction (PCR).

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FIG. 1.

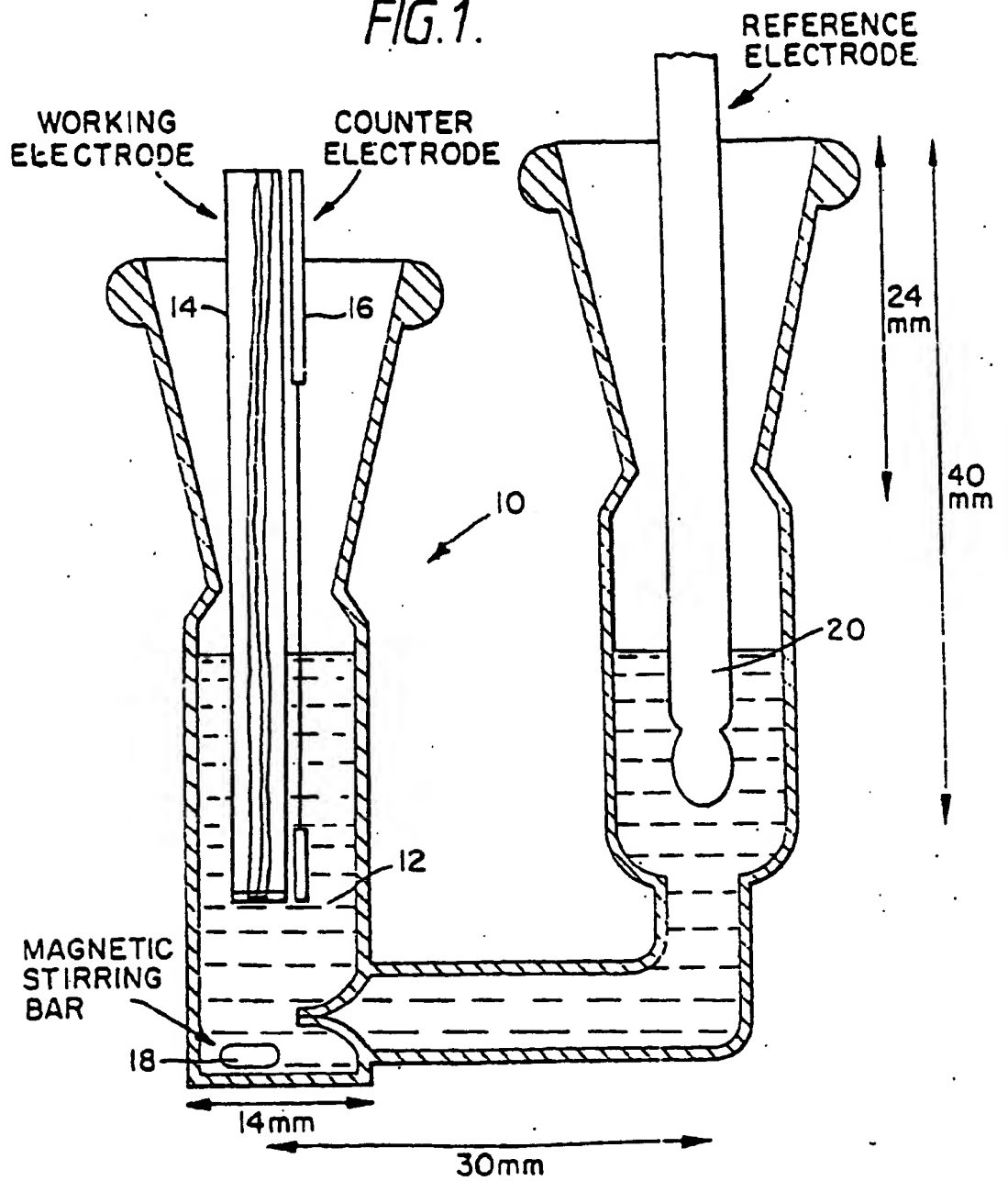
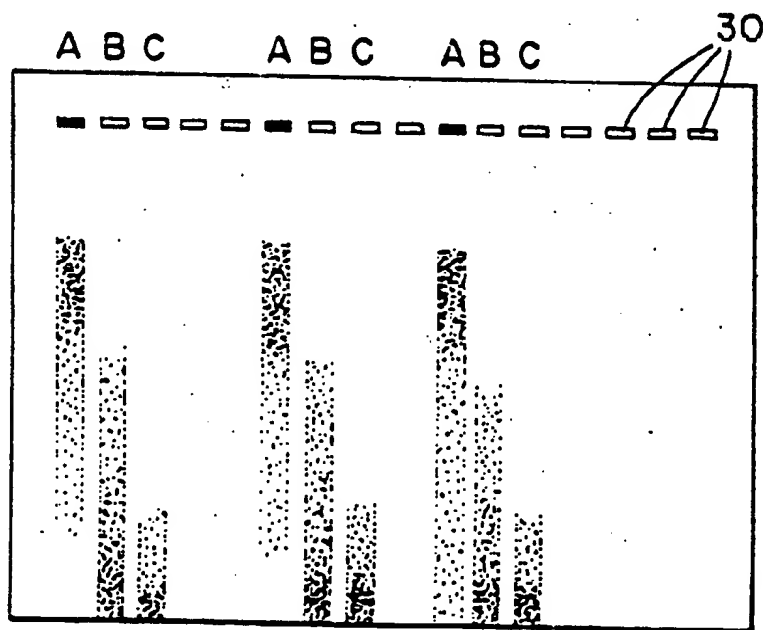


FIG. 2.



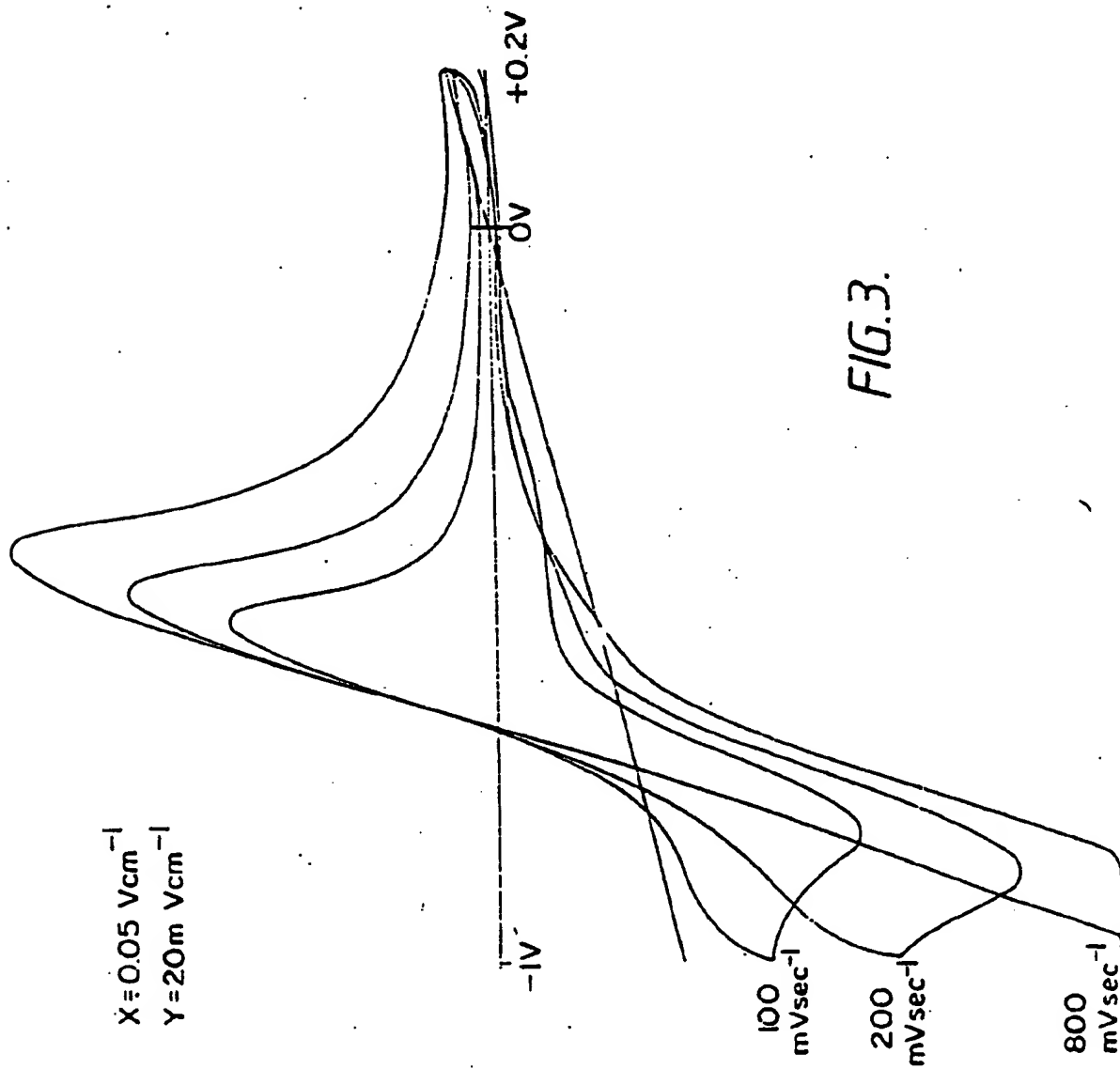


FIG. 3.

# TREATMENT OF NUCLEOTIDE MATERIAL

This invention relates to a process for the treatment of nucleotide material.

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US Patent No 4683195 (Kary B Mullis et al, assigned to Cetus Corporation) discloses a process for amplifying and detecting a target nucleic acid sequence contained in a nucleic acid or mixture thereof by separating the complementary strands of the nucleic acid in the presence of oligonucleotide primers, extending the primers to form complementary primer extension products and then using those extension products for the synthesis of the desired nucleic acid sequence. The individual process steps can be carried out repetitively to generate large quantities of the nucleic acid sequence that may be required to be reproduced from even a single strand of the starting material. However, the step of separating the complementary strands of the nucleic acid is by thermal denaturation, and the need for thermal cycling limits the speed at which the multiplication process can be carried out, requires use of special heat resistant enzymes for the extension step, and can lead to evaporative loss of water.

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It is therefore an object of the invention to provide a process for converting double stranded nucleic acids into single stranded nucleic acids which can be carried out at ambient or near ambient temperatures.

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According to the invention double stranded nucleic acid may be converted into denatured single stranded nucleic acid in free solution by applying an electrical potential to a solution containing the nucleic acid.

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H. W. Nurnberg, "Applications of Advanced Voltammetric Methods in Electrochemistry" [H. W. Nurnberg et al (Editors), "Bioelectrochemistry", Plenum Inc (New York), 1983, pp. 183-225] discloses partial helix opening of adsorbed regions of native DNA to form a so-called ladder structure. However, the DNA is effectively bound to the electrode surface and separation of the individual strands to provide useful lengths of single stranded DNA in solution has not been demonstrated. The denatured single stranded DNA reported by Nurnberg either has reorientation of adsorbed bases or is chemically modified by reduction of adenine and cytosine in the single stranded regions.

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F. Jelen and E. Paleck, "Nucleotide Sequence-Dependent Opening of Double-Stranded DNA at an Electrically Charged Surface", Gen. Physiol. Biophys., (1985), 4, 219-237 discloses the opening of a DNA double helix on prolonged contact of the DNA molecules with the surface of a mercury electrode. The mechanism of opening of the helix is postulated to be anchoring of the polynucleotide chain via hydrophobic bases to the electrode surface after which the negatively charged phosphate residues of the DNA are strongly repelled from the electrode surface at an applied potential close to -1.2 volts, the strand separation being brought about as a result of the electric field adjacent to the electrode. There is no disclosure of separating the strands of the DNA double helix while the DNA is in solution rather than adsorbed onto the electrode, and there is no disclosure of single strand DNA in solution as a result of this treatment.

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N. L. Palecek, "860-Electrochemical Behaviour of Biological Macromolecules", Bioelectrochemistry and Bioenergetics, 15, (1986), 275-295 discloses a peak in voltammogram measurements of DNA at a about -1.8 volts corresponding to chemical reduction of guanine to dihydroguanine, but this reduction does not

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require opening of the double helix into its individual strands.

5 The process of the invention is believed, although the invention is not limited to this theory, to proceed by an electron transfer to the DNA while the DNA is in solution, the electron then weakening the hydrogen bonds between adenine and thymine or cytosine and guanine which can bring about the separation of the strands. The electron transfer is believed to be reversible, and there is no chemical modification of the DNA molecule itself or its individual strands, the strand separation or denaturing process being reversible and the strands being capable of re-hybridising in so far as re-hybridisation is not prevented by other unrelated factors of the DNA molecule itself or of the solution in which it is present. If, for example, the process is carried out at or adjacent to the pre-melting temperature of the DNA (i.e. the temperature at which the hydrogen bonds between adenine and thymine break and the cytosine-guanine bonds are ready to break but have not yet broken), the material may then not re-hybridize simply on removal of the voltage from the electrode.

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Re-hybridisation will also, of course, not proceed if the temperature is too low.

5 The strand separation is brought about by an electron transfer process to DNA that is free in the solution rather than adjacent to and adsorbed onto an electrode that process preferably being the result of the presence in the solution containing the DNA of a mediator which receives electrons from  
10 the electrode and transfers them to the DNA to effect said strand separation. For the present purposes a mediator is defined to be an inorganic or organic molecule which is capable of reversible electron transfer at an electrode and which passes  
15 electrons on to or receives electrons from a biological molecule, in this instance the nucleic acid present in solution. Such mediators are described by M. L. Fultz et al, "Mediator compounds for the electrochemical study of  
20 biological redox systems: a compilation", *Analytica Chimica Acta*, 140 (1982), 1-18. The mediator should be soluble in the solvent for the DNA (which may be water or a solvent other than water) and compounds having a redox potential of 0 to -2 volts, preferably -0.2 to -1 volt and especially about  
25 -0.4 volts are preferred. Thus the mediator may be

a water or solvent soluble compound having conjugated or aromatic groups and one or more hetero-atoms and may be a compound of the quinone or bipyridyl series, especially a viologen such as methylviologen or a salt thereof. The choice of mediator is not believed to be critical provided that its redox potential is within the required voltage range and compound does not otherwise affect or interfere with DNA or other materials present in the solution such as enzymes or oligonucleotide probes. The use of a mediator enables the DNA or other nucleic acid material to be denatured into its individual strands at an applied voltage of -0.1 volt or less. Although denaturation has been observed by the present inventors at a voltage of -1 volt, it is believed that this may be an overvoltage and the voltage needed to bring about actual denaturation may be as low as -0.8 volts especially since the redox potential of the mediator is typically 0.4 volts. The experiments reported herein have been carried out at negative electrode voltages, but the possibility of bringing about denaturation at positive electrode voltages by an electron transfer process in which electrons are removed from the DNA can not be excluded. In principle the application of an electric charge to

native DNA or similar nucleic acid material can bring about separation of the individual strands whether that charge is a positive or a negative electrical charge, the mechanism of separation being postulated to be the electrical repulsion of like charges injected or transferred into the nucleic acid material.

The process may be carried out in a cell in which there is a working electrode and a counter electrode. The working electrode may be of any convenient material e.g. a noble metal such as silver, gold or platinum, or it may be a carbon electrode. The electrode may be a so called "modified electrode" in which the electron transfer is e.g. by an electron donating or electron accepting compound such as a mediator coated onto, or adsorbed onto, the surface of the electrode which is otherwise of an inert material. The electron transfer may also be from or to an electrode consisting at least partially of a mediator compound e.g. formed wholly of the mediator compound. In a practical cell the working, counter and reference electrodes may be formed on a single surface e.g. a flat surface by e.g. thick film screen printing, ink jet printing, or by using a photo-resist followed by

etching. Electrode arrays may be provided for multiple DNA experiments.

5 The strand separation may be carried out in an aqueous medium or in a solvent other than water, for example a mixture of water with an organic solvent such as tetrahydrofuran, alcohol or acetone, or it may be carried out in a polar organic solvent e.g. acetonitrile. The use of non-polar solvents is not  
10 at present excluded, but is not preferred. The process may be carried out at temperatures less than 100°C e.g. ambient temperatures or if desired temperatures adjacent the pre-melting temperature of the DNA or other biologically active compound e.g.  
15 at temperatures of about 60°C. The process may be carried out pH's of from 3 to 10 conveniently about 5.5. The DNA may be dissolved in an aqueous solution containing a buffer whose nature and ionic strength are such as not to interfere with the  
20 strand separation of the DNA. The strand separation reaction may take up to 24 hours or more depending upon the nature of the DNA sample and the mediator employed. Strand separation is believed to have been observed in the absence of mediator but  
25 proceeds more slowly.

It is preferred that the process of strand separation should be carried out in a cell in the absence of stirring or under gentle stirring conditions. This not only assists in preventing shearing of the DNA but also is believed to facilitate electron transfer to the DNA with a low degree of entrapment of the DNA on the electrode surface. The reason is that the mediator being a much smaller molecule than the DNA diffuses rapidly through the solution compared to the DNA so that the transport of the reduced form of the mediator from the electrode surface to the DNA molecule is a rapid process compared with the diffusion of the DNA molecule towards the electrode. In a case of a negatively charged electrode, the mediator will transfer electrons to the DNA which will itself acquire a negative charge and therefore will tend not to reach the surface of the electrode but to be maintained in solution. It is therefore possible that the process could be carried out in a two compartment cell divided by a membrane, the DNA solution being in a compartment remote from the cathode, and the electrons being transferred from the cathode to the DNA by the mediator which the cell membrane is permeable.

The process of the invention is believed to be industrially applicable because the single strand DNA or biologically material is useful in its own right. For example, it may be used to provide a  
5 stock of single stranded DNA material for hybridisation studies during research and as a molecular weight marker for gel electrophoresis testing.

10 It may also be used for hybridisation studies with an indicator probe to identify a gene sequence e.g. specific to a particular organism or specific to a particular hereditary disease of which sickle cell anaemia is an example. Thus invention provides a  
15 process for detecting the presence or absence of a predefined nucleic acid sequence in DNA or other nucleotide material which comprises:

denaturing a sample of DNA under a voltage supplied  
20 to the sample DNA in solution by means of electrode;

hybridising the denatured DNA with an oligonucleotide probe for the sequence; and

25 determining whether the hybridisation has

occurred. The determination may be by means of a radio-labelled, optically labelled or enzyme-labelled probe.

5 The standard procedure for carrying out a DNA probe assay involves the preparation of double-stranded DNA from the sample, its denaturation by heat or alkali or both, the binding of the resulting single stranded material to a solid support e.g. a  
10 filter, addition of a labelled probe for the sequence of interest and hybridising at 25 to 45°C, optionally in the presence of organic solvent such as dimethyl formamide, washing away excess labelled probe and detecting the label. This is a complex  
15 and time-consuming process. One improved method for carrying out DNA probes is the so called "sandwich" technique where a specific oligonucleotide is immobilised on a plastic surface. The surface having the specific oligonucleotide thereon is then  
20 hybridised with a solution containing the target DNA in a single-stranded form, after which a second labelled oligonucleotide is then added which also hybridises to the target DNA. The plastic surface is then washed to remove unbound labelled  
25 oligonucleotide, after which any label which has

become bound to target DNA on the surface can be detected later. This procedure can be simplified by using the present process to denature the double-stranded DNA into the required single-stranded DNA. The working electrode and mediator can be incorporated into a test tube or a well in which the DNA probe assay is to be carried out. The DNA sample and oligonucleotide primers can then be added, the reducing voltage applied to denature the DNA and then remove, the resulting single-stranded DNA is hybridised with the specific oligonucleotide immobilised on the plastic surface, after which the remaining stages of a sandwich assay are carried out. All the above steps can then take place without a need for high temperatures or addition of alkali reagents as in the conventional process.

For the so called "filter support" form of DNA probe assay, the target DNA may be bound to the filter which is then saturated with mediator, after which the filter may be physically applied to a working electrode (which may be a sheet or a dot array) after which a reducing voltage is applied to the electrode to denature the DNA in situ on the filter. Labelled oligonucleotide may then be added, after



which the filter is washed and any remaining labelled oligonucleotide is detected e.g. by fluorescence or by radio-active counting depending upon the nature of the label. It is also possible to apply just the appropriate voltage during hybridisation of the labelled probe to ensure binding thereof. In this way the requirement for baking of filters to achieve denaturation of DNA bound thereon is avoided - in fact it may be possible to avoid taking the assay away from room temperature when electrochemical denaturation is used.

A further use of electrochemical denaturation of DNA probes is in histochemistry where labelled DNA probes can be hybridised in situ to tissue sections on a microscope slide. This procedure is currently carried out by heating a tissue slice on the slide to a high temperature in order to denature the target DNA. According to the invention the target DNA may be denatured electrochemically by soaking the tissue slice in a solution containing a mediator and applying a reducing voltage e.g. by using an electrode incorporated into or formed on the surface of the microscope slide itself.

The denaturing process of the invention may also be

used in gel electrophoresis. A gel of agarose or polyacrylamide containing double-stranded DNA (which may have been separated by charge or size) cannot be heated or treated with alkali to denature the DNA into its single strand because the gel collapses. Instead the gel may be soaked with a mediator, placed on a working electrode (a flat plate or array) after which a reducing voltage is applied to denature the DNA in the gel. The DNA in the gel can then be subjected to electrophoresis a second time, preferably to give a two-dimensional gel in which the first electrophoresis dimension is carried out with double-stranded DNA and the second electrophoresis dimension is carried out with single-stranded DNA or with single-stranded DNA which has been hybridised with a labelled oligonucleotide probe to enable specific sequences to be detected. The possibility of carrying two-dimensional gel electrophoresis of DNA, the first dimension corresponding to double-stranded DNA and the orthogonal dimension corresponding to single-stranded DNA, could provide a new technique for DNA analysis permitting the detection of component single strands of unequal length in double-stranded pieces of DNA. The ability to denature the DNA within the gel and to hybridise the

resulting single-stranded DNA directly with labelled oligonucleotides is an advantage because it avoids the need to remove the appropriate portions of the gel and separate the double-stranded DNA therefrom before the denaturing and hybridisation can be carried out.

This process can be further expanded to achieve three-dimensional electrophoresis of DNA, that is movement in all three planes within the gel. An electrode can be used to draw probe sequences into a gel from the surface above the separated samples. This can be achieved because large DNA sequences will move very slowly through the gel towards the electrode, but small sequences move much more quickly and could overtake and hybridise with the larger sequences.

The invention also has application in cytology because fragile materials such as cells can be analysed for the presence of specific sequences in their DNA by making the cells permeable to mediator if that is not already the case, applying a reducing voltage to denature the genomic, plasmid, mitochondrial or chloroplast DNA, having a labelled oligonucleotide probe to the mediator permeable

cells and causing the probe to hybridise with the  
denatured DNA to enable particular sequences to be  
detected. Excess probe and mediator can be removed  
by dialysis and the cells can then be sorted and  
5 analysed by flow cytometry and/or by microscope  
examination. The fragile structures of the cell can  
be preserved during this method whereas the heat or  
alkali required for conventional methods of DNA  
denaturing would cause irreversable changes in the  
10 cellular appearance.

The invention may also be used as one step in the  
amplification of viral or DNA vectors. These  
vectors commonly exist in their native form as  
15 single-stranded DNA, and exist in the cell cultures  
in intracellular form as a double stranded DNA.  
Lysis of the cells to recover the viral DNA which  
has grown gives rise to the double stranded form of  
the viral DNA which has to be converted back into  
20 the single stranded native form of the virus. The  
process of the invention may be used to carry out  
that step.

The invention may further be used for gene  
25 replication in a process analogous to the existing  
polymerase chain reaction (PCR) for replicating DNA.

Thus in a further aspect the invention provides a process for replicating DNA which comprises:

5 separating the strands of a sample DNA in solution under the influence of an electrical voltage applied to the solution from an electrode;

10 hybridising the DNA with at least one oligonucleotide primer (see US Patent 4683195) that hybridises with at least one of the strands of the denatured DNA;

15 synthesising an extension product of the or each primer which is sufficiently complementary to the respective strand of the nucleic acid to hybridise therewith (see also US Patent 4683195); and

20 separating the strand of an extended DNA from the nucleic acid strand with which it is hybridised to obtain the extension product.

25 In the above process the hybridisation step may be carried out using a single primer that hybridises with only one of the strands, but for amplification it is desirable to use two primers which are complementary to the different strands of the DNA.

The denaturation to obtain the extension products can be carried out by applying to a solution of the extension product a voltage from the electrode. The process may be carried out stepwise as in the  
5 existing thermal method of PCR, but it may also be carried out continuously because as soon as the extension product is formed it can be broken down by the electrode voltage so that there is no need to wait for complete separation of the strands of the  
10 original sample before the separation of the extension products can begin. Thus, provided that the primer will hybridise with the DNA a denaturation product will grow, and the technique can be used analytically to determine in a very  
15 small sample the DNA e.g. a single strand in the presence of a particular nucleotide sequence which is then amplified into quantities sufficient for bulk analysis.

20 The invention will now be described with reference to the following drawings and examples.

Figure 1 is a diagram of a test cell;

Figure 2 is a drawing of a developed electrophoresis gel plate showing the movement of single and double stranded DNA, the forms of DNA being firstly a stock DNA, secondly an electrically denatured DNA according to the invention and thirdly a thermally denatured DNA;

Figure 3 is a voltammogram obtained using a solution of DNA electrically denatured into its individual strands according to the invention; and

The invention is illustrated by the following example.

#### Example

In Figure 1 there is shown a cell structure 10 comprising the working compartment 12 in which there is a body of DNA-containing solution, a working electrode 14, a counter electrode 16 and a magnetic stirrer 18. A reference electrode 20 in a separate side arm is connected via a "luggin" capillary to the solution in the sample 12. The working electrode, counter electrode and reference electrode are connected together in a potentiostat arrangement so that a constant voltage is maintained between the

working electrode 14 and the reference electrode 20,  
the counter-electrode 16 controlled by an  
amplifier feedback loop to maintain thus set  
potential. Such potentiostat arrangements are well  
5 known.

1.6 of a solution in distilled water of  
methylviologen dichloride, the solution containing  
1mg of that compound per ml was filled into working  
10 compartment of the electrochemical cell. The  
reference arm of the cell containing the electrode  
20 contained 0.4ml of this solution. A sample of  
120 microlitres of a stock solution of calf thymus  
DNA (Sigma Chemical Company D4522, average molecular  
15 weight 5Kb) containing 1mg per ml was added to the  
working chamber and a voltage of -1.0 volts was  
applied between the working electrode 14 and the  
reference electrode 20. The cell was left overnight  
without stirring. A blue colour from reduced  
20 viologen was visible in the immediate vicinity of  
the working electrode 14. The cell was left  
unstirred because it was considered that the  
diffusion rate of the viologen would be much  
greater than that of the double stranded DNA so that  
25 the viologen might transfer electrons to the DNA  
without the DNA itself contracting the electrode 14



and becoming bound thereto. This would reduce the possibility of irreversible coating of the electrode. A 100 microlitre sample of the material in the working part of the cell was taken at the end of this period and prepared for gel electrophoresis analysis by mixture with 20 microlitres of gel loading buffer which contained 0.25 per cent w/v of bromophenol blue, 0.25 per cent w/v xylene cyanol, and 30 per cent v/v of glycerol, the balance being water. The resulting DNA solutions were loaded in 10 microlitre wells formed in an electrophoresis gel. The gel (Figure 2) had a number of wells 30 into which the samples could be inserted, and nine 10 microlitre samples were placed into individual wells. The gel had a total volume of 30ml and was 10cm wide and 7.5cm long; it was 0.5 per cent w/v agarose in TBE buffer containing 0.089 M tris(hydroxymethyl)methyl ammonium borate and 0.01 M of EDTA, the gel having a pH of 8. The gels were incubated for 80 to 90 minutes at an applied voltage of 55 volts and then stained by addition of 0.75 ml of ethidium bromide (2.0 mg/ml). The stained gels were trans-illuminated with uv light and photographed using a red filter to reduce background from the uv source. The appearance of the resulting gel is shown in Figure 2.

Applied to each electrophoresis gel were three sets of samples A, B and C to give the nine test solutions referred to above. Sample A was the starting material used in the test with calf thymus DNA. Sample B was a sample of the calf thymus DNA which had been electrically denatured according to the invention and sample C was a sample of thermally denatured single stranded DNA. It will be noted that the samples B and C are of high mobility through the gel indicating that the DNA in the cell had been electrically denatured into single strands.

The above experiment was repeated as before, but samples were taken after 1.5 hours, 3 hours and 22 hours. A gradual progressive denaturation of the DNA into single strands was observed. After 1.5 hours there was a mixture of partially and fully denatured DNA but no evidence of wholly native DNA, and in later samples the proportion of fully denatured DNA increased upto 22 hours.

Figure 3 is a cyclic voltammogram of the material from the cell after 22 hours. The positive peak at -0.45 volts was attributed to the reduction of the

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methyl viologen mediator and demonstrates that electron transfer is taking place.

CLAIMS

1. A process for converting double stranded DNA into single stranded DNA which comprises applying an electrical potential to a solution containing the DNA to separate the strands thereof.
2. A process according to claim 1, wherein the strand separation is brought about by an electron transfer process within the solution of the DNA.
3. A process according to claim 2, wherein the solution in which an electrode is present contains DNA together with a compound which receives electrons from the electrode and transfers them to the DNA to effect said strand separation.
4. A process according to claim 3, wherein the compound has a redox potential of 0 to -2 volts.
5. A process according to claim 4, wherein the compound has a redox potential of -0.2 to -1 volt.
6. A process according to any of claims 3 to 5, wherein the strand separation is carried out using an aqueous solution of the DNA and compound, and

the compound is methyl viologen, benzyl viologen, neutral red or phenosafranin or a salt thereof.

5        7. A process according any preceding claim, wherein the electrode is of carbon, silver, gold or platinum.

10       8. A process according to claim 1 or 2, wherein the electron transfer is by an electron donating compound coated or adsorbed onto the surface of an otherwise inert electrode.

15       9. A process according to claim 1 or 2, wherein the electron transfer is from an electrode consisting at least partly of a compound that undergoes a redox reaction and which can transfer electrons to DNA as a result of said reaction.

20       10. A process according to any of claims 1 to 5, wherein the strand separation is carried in a solvent other than water.

25       11. A process according to any preceding claim, carried out at a temperature less than 100°C.

12. A process according to any preceding claim carried out at ambient temperatures.

5 13. A process according to any of claims 1 to 11, carried out at the pre-melting temperature of the DNA.

14. A process according to any preceding claim carried out at the pH of 3 to 10.

10 15. A process according to any of claims 1 to 12 carried out a pH of about 5.5.

15 16. A process according to any preceding claim, wherein the DNA is dissolved in an aqueous solution containing a buffer whose nature and ionic strength are such as not to interfere with strand separation of the DNA.

20 17. A process according to claim 16, wherein the DNA is dissolved in an aqueous solution whose ionic strength is 5mM or less.

25 18. A process for replicating DNA which comprises:

separating the strands of a sample DNA in solution under the influence of an electrical voltage applied to the solution from an electrode;

5 hybridizing the DNA with at least one oligonucleotide primer that hybridises with at least one of the strands of the denatured DNA;

10 synthesising an extension product of the or each primer which is sufficiently complementary to the respective strand of the nucleic acid to hybridize therewith; and

15 separating the strand or extended DNA from the nucleic acid strand with which it is hybridised to obtain the extension product.

19. A process according to claim 18, wherein the hybridization step is carried out using two primers  
20 which are complementary to different strands of the DNA.

20. A process according to claim 18 or 19, wherein the denaturation to obtain the extension products is  
25 carried out by applying to a solution of the extension product a voltage from an electrode.

21. A process for detecting the presence or absence of a predetermined nucleic acid sequence in DNA which comprises:

5       denaturing a sample of DNA under a voltage applied to the sample in solution by means of an electrode;

10       hybridizing the denatured DNA with an oligonucleotide probe for the sequence; and

determining whether the said hybridization has occurred.



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<b>(54) Title: IMPROVED SANDWICH HYBRIDISATION TECHNIQUE FOR THE DETECTION OF NUCLEOTIDE SEQUENCES</b>  <b>(57) Abstract</b>  An improved sandwich hybridisation diagnostic method in which there is used as the immobilised polynucleotide, a single-stranded polynucleotide sequence covalently coupled to particles or beads of a synthetic resin, preferably a cross-linked macroporous cellulosic resin. Also disclosed is a particular application of the method to the detection of the abnormal human (sickle cell) $\beta$ -globin gene and diagnostic kits for use therein.		

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IMPROVED SANDWICH HYBRIDISATION TECHNIQUE  
FOR THE DETECTION OF NUCLEOTIDE SEQUENCES

FIELD OF INVENTION

- 5        This invention relates to the detection of nucleotide sequences, including both DNA and RNA sequences.

BACKGROUND AND PRIOR ART

- 10        The ability to detect the presence (or absence) of a given RNA or DNA sequence in a sample, e.g. a clinically obtained sample or specimen, is potentially of great benefit to mankind. Existing methods of detection are, however, extremely time consuming and labour intensive and are therefore for use on anything other than a very limited scale. In many cases also sensitivity is undesirably low. In one commonly used procedure, for example,
- 15        specific DNA sequences are detected in complex mixtures by digesting the sample with an appropriate restriction endonuclease, followed by size fractionation by gel electrophoresis, transfer of the sized fractions from the gel to a nitrocellulose or other suitable membrane, hybridisation to a radioactively labelled probe, usually a <sup>32</sup>P-labelled probe, and finally auto-
- 20        radiography. Such a procedure is inherently cumbersome and unsuited for automation. Also, very often the quantity of DNA or other nucleotide sequence to be detected and which is transferred onto the membrane is so small that in order to obtain a detectable signal after hybridisation a highly radioactive probe has to be used, which is disadvantageous both from the
- 25        handling point of view and from the point of view that the radioactive label of choice, i.e. <sup>32</sup>P, has a relatively short half-life making it unsuitable for use as a stock reagent.

- 30        An alternative to the above is the sandwich hybridisation technique disclosed by Dunn A.R. and Hassell J.A. in (1977) Cell 12 23-26 in which the sample containing the nucleotide sequence to be detected is contacted with a complementary fragment immobilised on a membrane, e.g. a nitrocellulose filter, and to which the sequence to be detected hybridises in a first hybridisation step leaving an unhybridised tail available for a second hybridisation step with a labelled probe.

- 35        In EP-0079139 a proposal is made for the utilisation of the above described sandwich hybridisation technique in the identification of micro-

organisms in a sample. In accordance with that proposal a sample containing a single-stranded nucleic acid sequence from the micro-organism to be detected is contacted with two single-stranded fragments obtained from the genome of the micro-organism in question either directly or by recombinant DNA technology and complementary to different positions of the sequence to be detected, but not to each other, the one being immobilised on a solid carrier, preferably a nitrocellulose filter, and the other being labelled. Although labelling with labels other than radioactive labels is implied, only radioactive labelling is disclosed, and specifically labelling with  $^{125}\text{I}$ . In the presence of the single-stranded nucleic acid sequence to be detected, which hybridises with both the immobilised fragment and the labelled fragment, the labelled fragment becomes bound to the carrier and can be detected thereon by autoradiography, thus giving rise to positive identification of the micro-organism from which that single-stranded sequence originated.

Whilst the sandwich hybridisation technique provides advantages over the first described procedure inherent disadvantages remain arising particularly from the finite and quite limited quantity of nucleic acid which can be immobilised onto the nitrocellulose filter or other membrane. This in particular places limits on both the speed and sensitivity of the detection method. Also it appears that the efficiency of the hybridisation particularly in the second stage is very low, thus reducing the sensitivity of the method still further.

In EP-0070687 a hybridisation diagnostic method is disclosed which uses light-labelled single-stranded polynucleotide reagents for hybridising with immobilised sample single-stranded polynucleotides. A variety of solid supports are suggested for the immobilised sample single-stranded polynucleotide including activated glass beads, polyacrylamide, agarose or sephadex beads, and cellulose. Various known methods are also suggested for coupling the sample polynucleotide to the support, largely by reference to published literature, e.g. Methods in Enzymology Vol. XXXIV, Part B, 463-475, 1974 and Vol. XLIV, 859-886, 1976, but no specific examples of an operative method are given. It is also suggested that the sample single-stranded polynucleotide can be immobilised by contacting the sample under hybridisation conditions with an immobilised first single-stranded polynucleotide reagent complementary to a different and mutually exclusive portion of the sample polynucleotide to that which is required for the

hybridisation of the light-labelled polynucleotide reagent, i.e. a so-called sandwich hybridisation procedure. Again no specific examples are given of the procedure, or of the method by which the first polynucleotide reagent is attached to the support in the first place.

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#### SUMMARY OF THE INVENTION

In accordance with one aspect of the present invention we have discovered that both the speed and efficiency of the sandwich hybridisation reaction, particularly the second stage, can be substantially increased by using solid particles or beads of a macroporous resin as the carrier for the immobilised nucleic acid fragment thus providing a method for the detection of nucleic acid fragments which is capable of providing a much improved sensitivity. In particular the use of particles or beads of a cross-linked macroporous resin such as sephacryl appears to result in substantially improved covalent bonding of the polynucleotide reagent to the support.

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In a second aspect of this invention, a particularly sensitive method has been developed for the detection of the abnormal human (sickle cell)  $\beta$ -globin gene by the sandwich hybridisation of a Dde I digest of the abnormal gene with an immobilised polynucleotide reagent covalently bonded to solid resin particles or beads, and a second labelled polynucleotide reagent, such reagents comprising polynucleotide sequences obtained by restriction endonuclease treatment of the normal gene and complementary to different portions of a restriction fragment contained within the digest and containing the single base change in the sixth codon of the  $\beta$ -globin gene that is characteristic of the abnormal (sickle cell)  $\beta$ -globin gene.

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In accordance with the present invention therefore, there is provided a method for the detection of a given nucleic acid sequence in a sample, which comprises contacting the sample under hybridisation conditions with a first reagent comprising solid particles or beads having immobilised thereon a single-stranded nucleic acid fragment comprising a sequence complementary to a portion of the sequence to be detected and with a second reagent comprising a labelled single-stranded nucleic acid fragment comprising a sequence complementary to a different portion of the sequence to be detected, but non-complementary to the immobilised fragment, thereby forming a hybridisation duplex comprising the sequence to be detected hybridised with both reagents, separating the hybridisation duplex from any

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remaining unbound label, and detecting the presence, if any, of label bound to the hybridisation duplex.

#### DETAILED DESCRIPTION

5       The first reagent used in the method of this invention preferably comprises a single-stranded nucleic acid fragment immobilised onto the solid particles or beads by covalent bonding between reactive, e.g. amino, groups in the nucleic acid fragment, and reactive groups in or on the solid particle. To this end the preferred solid particles used in the method of this invention  
10       are polymer beads formed from a natural or synthetic polymer having reactive, e.g. amino or hydroxy, groups attached to the polymer chain. Most preferred are cellulosic polymer beads such as those sold under the trade names Sephadex and Cellex and more particularly macroporous cellulosic materials such as Sepharose and Sephacryl.

15       Various methods exist and are known for the covalent bonding of nucleic acid fragments to such polymer beads, including coupling with carbodiimides via terminal phosphate groups and coupling via cyanogen bromide. Particular mention may be made, however, of coupling via diazotisation of aromatic amino groups attached to the polymer matrix, which technique is  
20       disclosed in detail in (1982) Nucleic Acid Res. 10, 22, 1799-1810 and 7163-7196.

Preferably the polymer beads will have a particle size in the range 5 to 50 microns, more preferably 10 to 20 microns.

25       Likewise various methods exist and are known for the labelling of single-stranded nucleic acid fragments to provide the second reagent used in the method of this invention. Labelling with radioactive isotopes such as <sup>32</sup>P and <sup>125</sup>P has already been mentioned and can be used in the present invention. However, it is envisaged that, because of the greater sensitivity that can be achieved by the present invention, less highly radioactive isotopes  
30       can be used as the label and in particular tritium. Also it is envisaged that non-radioactive labels e.g. enzymatic, fluorescent and chemiluminescent labels will be suitable, and in many cases may be preferred.

Once the reagents have been prepared, and it is envisaged that these will be available in kit form, the method of the invention is extremely simple  
35       to carry out and is readily adaptable to automatic or semi-automatic equipment. Essentially it is necessary merely to mix the sample with the two



reagents, either simultaneously or successively, under conditions effective to permit formation of the hybridised duplex comprising the two reagents hybridised to the restriction fragment which is to be detected. The hybridisation is rapid, and since in contrast to the sandwich hybridisation technique of EP-0079139, both the first (immobilised) reagent and the second (labelled) reagent can be used to infinite excess, merely by increasing the quantity used, the amount of bound label can be maximised thereby to provide a maximum signal for subsequent detection. Thus the method of the present invention can be of great sensitivity, this being determined not by the concentration of the reagents, which effectively can be infinite, but by the concentration of the sequence to be detected in the sample. This is in marked contrast to the method of EP-0079139 where the concentration of the immobilised reagent is finite, and relatively low, so that the quantity of label bound to the duplex is equally low and is determined not by the concentration in the sample of the sequence to be detected, which is usually far in excess of the quantity of the immobilised reagent, but by the quantity of the immobilised agent. In the method of the present invention a far higher proportion of the sequence to be detected present in the sample is hybridised and consequently labelled. Not only that, but as already indicated, there is a substantial and surprising increase in the efficiency of the hybridisation reaction using beads as the carrier as opposed to a filter or membrane, e.g. of cellulose nitrate.

The sample used in the method of this invention can be a purified and/or fractionated sample containing the polynucleotide fragment or fragments to be identified in single-stranded form, but it is a major advantage of this invention that the sequence to be detected does not have to be separated from the sample prior to labelling as in the Southern blotting technique. It is therefore possible in accordance with the present invention to detect a given polynucleotide sequence in a crude mixture of polynucleotide fragments obtained by the digestion of the original sample, e.g. a clinical sample containing DNA or RNA, with an appropriate restriction endonuclease and subsequent denaturation to reduce the restriction fragments to single-stranded form.

Following hybridisation the polymer beads or other particles carrying the labelled hybridisation duplex are separated, e.g. by centrifuging and washing, from unbound excess label and the presence of bound label detected

in the appropriate manner, e.g. by a scintillation counter in the case of a radioactive label, or by enzymatic activity in the case of an enzyme label, or light detecting means in the case of a fluorescent or chemiluminescent label.

The method of the present invention has a wide range of applications arising from the ability rapidly to identify particular nucleic acid (DNA or RNA) sequences in a sample. Thus it can be used to identify pathogenic micro-organisms in a sample, genetic abnormalities, etc. as well as simply a gene mapping exercise. A particular application given by way of example is in the identification of sickle cell disease.

Sickle cell disease is caused by a single base mutation (A → T) in the sixth codon of the human  $\beta$ -globin gene. This change from GAG to GTG in the sixth codon, besides altering the properties of the resultant haemoglobin coded for by the gene with valine being incorporated into the protein rather than glutamine, also results in the loss of the Dde I and Mst II recognition sites at the 5' end of the  $\beta$ -globin gene. This is diagrammatically illustrated in the accompanying drawing which is a restriction map of Dde I and Hinf I sites at the 5' end of the  $\beta$ -globin gene. Dde I digestion of abnormal (sickle cell)  $\beta$ -globin gene will therefore produce a single 381 bp fragment comprising both Fragments B and C in a single continuous length, as opposed to two separate fragments, one of 201 bp (Fragment B) and 180 bp (Fragment C), that will be produced by Dde I digestion of the normal  $\beta$ -globin gene. Using the techniques already described to immobilise one of Fragment B or Fragment C onto polymer beads and to label the other, two reagents are provided capable of rapidly detecting the presence of abnormal sickle cell gene in a sample. Thus digestion of normal  $\beta$ -globin gene with Dde I will produce two fragments neither of which is capable of forming a "sandwich" with the two reagents. On the other hand, digestion of abnormal (sickle cell)  $\beta$ -globin gene with Dde I will produce a single fragment which is capable of forming a "sandwich" with the two reagents. When the Dde I digest is contacted with the two reagents under hybridisation conditions (i.e. after denaturation of the digest) the abnormal (sickle cell)  $\beta$ -globin gene will result in binding of the label, and hence a positive signal, whereas the normal  $\beta$ -globin gene will not.

In a practical experiment, the above procedure has been mimicked by digesting plasmid  $\beta$ F5 with Hinf I. Plasmid  $\beta$ F5 contains the 1.9 kb Bam HI restriction fragment of the human  $\beta$ -globin gene cloned into the single Bam

HI site of the vector pAT 153. Digestion of the plasmid  $\beta$ F5 with Hinf I produced a 341 kb fragment (Fragment A) which was recovered from the product of digestion by electrophoresis and electroelution.

5 After denaturation the 341 kb fragment was immobilised onto macroporous cellulosic polymer beads (Sephacryl S500) using the diazotisation method described by Seed B. in (1982) *Nucleic Acids Res.* 10, 1799-1810.

10 In a second operation plasmid  $\beta$ F5 was digested with Mst II to produce a 201 bp fragment (Fragment B, see Figure 1) which was likewise recovered from the digest. Fragment B was then labelled with  $^{32}\text{P}$  by nick translation using the technique of Rigby et al. (1977) *J. Mol. Biol.* 113, 237-251, to a specific activity of  $1.5 \times 10^7$  cpm  $\mu\text{g}^{-1}$ .

15 In two further operations plasmid  $\beta$ F5 was digested with Dde I and Bam HI. Digestion with Dde I produces short fragments not capable of "sandwich" hybridisation with Fragments A and B, whereas digestion with Bam HI produces one long fragment which is capable of "sandwich" hybridisation. In each case digestion was followed by denaturation to provide single-stranded DNA fragments.

20 Prior to sandwich hybridisation by the technique of the present invention the immobilised Fragment A was prehybridised overnight at  $65^\circ\text{C}$  in 1 ml. 40 mM PIPES pH 6.5, 1 mM EDTA 0.6 M NaCl, 0.1% SDS and  $250 \mu\text{g ml}^{-1}$  sonicated denatured salmon sperm DNA. Following prehybridisation, sandwich hybridisation was carried out by mixing either the denatured Dde I or Bam HI  $\beta$ F5 digests with immobilised 341 bp Fragment A and labelled 201 bp Fragment B in  $50 \mu\text{l}$  of the same buffer and allowing the mixture to stand  
25 overnight. The beads were then separated, washed three times for 10 minutes each at  $65^\circ$  with 1 ml of the same buffer but without the salmon sperm DNA and then counted. The results are shown in Table 1. These clearly show that with amounts of plasmid  $\beta$ F5 of 100 attomoles or over, a clear distinction can be drawn between the Bam HI digest and the Dde I  
30 digest. In the figures given in Table 1 the low background count obtained when either the "sandwich filling" was omitted or replaced by sonicated calf thymus DNA has not been subtracted.

Table 1

5	Amount of restricted	cpm on resin	cpm on resin
	$\beta$ F5 plasmid (attomoles)	when enzyme is Bam HI	when enzyme is Dde I
	10,000	552	63
	5,000	748	70
10	2,000	482	65
	1,000	266	71
	500	196	44
	100	107	62
	80	90	61
15	60	82	60
	40	62	57
	20	75	60

20 In a further practical experiment, the above sandwich hybridisation using the immobilised 341 bp Fragment A and the labelled 201 bp Fragment B has been repeated using a Dde I digest of 60 attomoles of human DNA both from a normal patient and from a homozygote sickle patient. The results were unequivocal. After background subtraction, a typical experiment gave

25 441 counts (10 minutes) for sickle DNA and 153 counts for normal DNA illustrating and confirming the effectiveness of the method of the invention in identifying the presence of sickle DNA.

By mixing the Bam HI and Dde I digests of  $\beta$ F5 it is possible also to mimic the case of the sickle heterozygote. In such an experiment, counts (10

30 minutes) were obtained using 100 attomoles plasmid DNA as follows: normal 128; heterozygote 296; homozygote 745; thus indicating that the method can be quantitated to identify heterozygotes as well as homozygotes.

In summary, the method of the present invention has the following advantages over present technology:

- 35 1. It is quicker. Electrophoresis, blotting and autoradiography are eliminated. The sandwich hybridisation can be carried out in as little as 8

hours; the separation of beads by gravity, centrifuge or magnetically takes seconds; and quantitation in a scintillation counter takes a few minutes. In practice results have been obtained within 48 hours of taking blood and the indications are that substantial reductions will be possible in the future.

- 5 2. It is extremely easy to automate and hence to process a large number of samples.
3. It is readily adaptable to other methods of DNA labelling.
- 10 4. It works as well with short restriction fragments as with longer ones and is readily adaptable for the use of oligonucleotides. This greatly enlarges the number of potential applications.
- 15 5. Whereas membranes have a (low) finite capacity for binding DNA, larger quantities of DNA can be readily handled in the present procedure by simply increasing the quantity of immobilised and labelled DNA's. This offers the possibility of either increasing the sensitivity of the assay or of using a labelled DNA of lower specific activity or of reducing the hybridisation time still further.

20 These advantages will now permit the rapid adoption of recombinant DNA technology to routine laboratories for the identification and characterisation of bacteria, viruses, and genetic disorders in humans, animals and plants.

CLAIMS

1. In a method for the detection of a given nucleotide sequence in a target polynucleotide, which comprises contacting the target polynucleotide under hybridisation conditions with (a) an immobilised polynucleotide comprising a single-stranded nucleotide sequence complementary to, and hybridisable under said conditions, with a first section of said sequence to be detected, and (b) a labelled polynucleotide probe comprising a single-stranded nucleotide sequence complementary to, and hybridisable under said conditions, with a second and different section of the sequence to be detected, labelled with a marker, thereby to form an immobilised hybridisation duplex comprising the immobilised polynucleotide, the target polynucleotide and the labelled polynucleotide, separating the immobilised duplex and determining the presence of the marker thereon, the improvement which comprises using as said immobilised polynucleotide, a polynucleotide containing said single-stranded complementary nucleotide sequence covalently bonded to solid particles or beads of a cross-linked macroporous resin.
2. In a method for the detection of a given nucleotide sequence in a target polynucleotide, which comprises contacting the target polynucleotide under hybridisation conditions with (a) an immobilised polynucleotide comprising a single-stranded nucleotide sequence complementary to, and hybridisable under said conditions, with a first section of said sequence to be detected, and (b) a labelled polynucleotide probe comprising a single-stranded nucleotide sequence complementary to, and hybridisable under said conditions, with a second and different section of the sequence to be detected, labelled with a marker, thereby to form an immobilised hybridisation duplex comprising the immobilised polynucleotide, the target polynucleotide and the labelled polynucleotide, separating the immobilised duplex and determining the presence of the marker thereon, the improvement which comprises using as said immobilised polynucleotide, a polynucleotide containing said single-stranded complementary nucleotide sequence covalently bonded to solid particles or beads of a cross-linked macroporous cellulosic material, said particles or beads having a particle or bead size in the range 5 to 50  $\mu$ .
3. A method according to claim 2, wherein the immobilised polynucleotide

is covalently bonded to the resin particles or beads by a diazotisation reaction between reactive groups on the resin and free amino groups on the polynucleotide.

- 5      4.    A method according to claim 2, wherein the immobilised polynucleotide is covalently bonded to the resin particles or beads by carbodiimide coupling between the phosphate groups at the 5' end of the polynucleotide and reactive groups on the resin.
- 10     5.    A method according to claim 2, wherein the polynucleotide probe comprises a radioactive marker or label.
6.    A method according to claim 2, wherein the polynucleotide probe comprises an enzyme marker or label.
- 15     7.    A method according to claim 2, wherein the immobilised polynucleotide and the polynucleotide probe are restriction endonuclease polynucleotide fragments.
- 20     8.    A method for the detection of the abnormal human (sickle cell)  $\beta$ -globin gene, which comprises digesting the gene with a Dde I restriction endonuclease to produce a restriction digest containing as a component a 381 bp restriction fragment containing the abnormal sixth codon which is a characteristic of the abnormal gene, contacting the digest containing that fragment
- 25     under hybridisation conditions with (a) an immobilised single-stranded polynucleotide covalently bonded to solid resin particles or beads, said single-stranded polynucleotide being hybridisable with a first section of said fragment, and (b) with a labelled single-stranded polynucleotide probe hybridisable with a second section of said fragment, wherein one of said single-
- 30     stranded polynucleotide probe and said labelled single-stranded polynucleotide is or comprises a 180 bp polynucleotide sequence obtained or obtainable by Dde I digestion of normal human  $\beta$ -globin gene and complementary to a first section of said 381 bp fragment, and the other is or comprises a 201 bp fragment obtained or obtainable by Dde I digestion of normal  $\beta$ -globin gene and complementary to a second, different, section of said 381 bp fragment,
- 35     separating the resulting immobilised hybridisation duplex comprising the

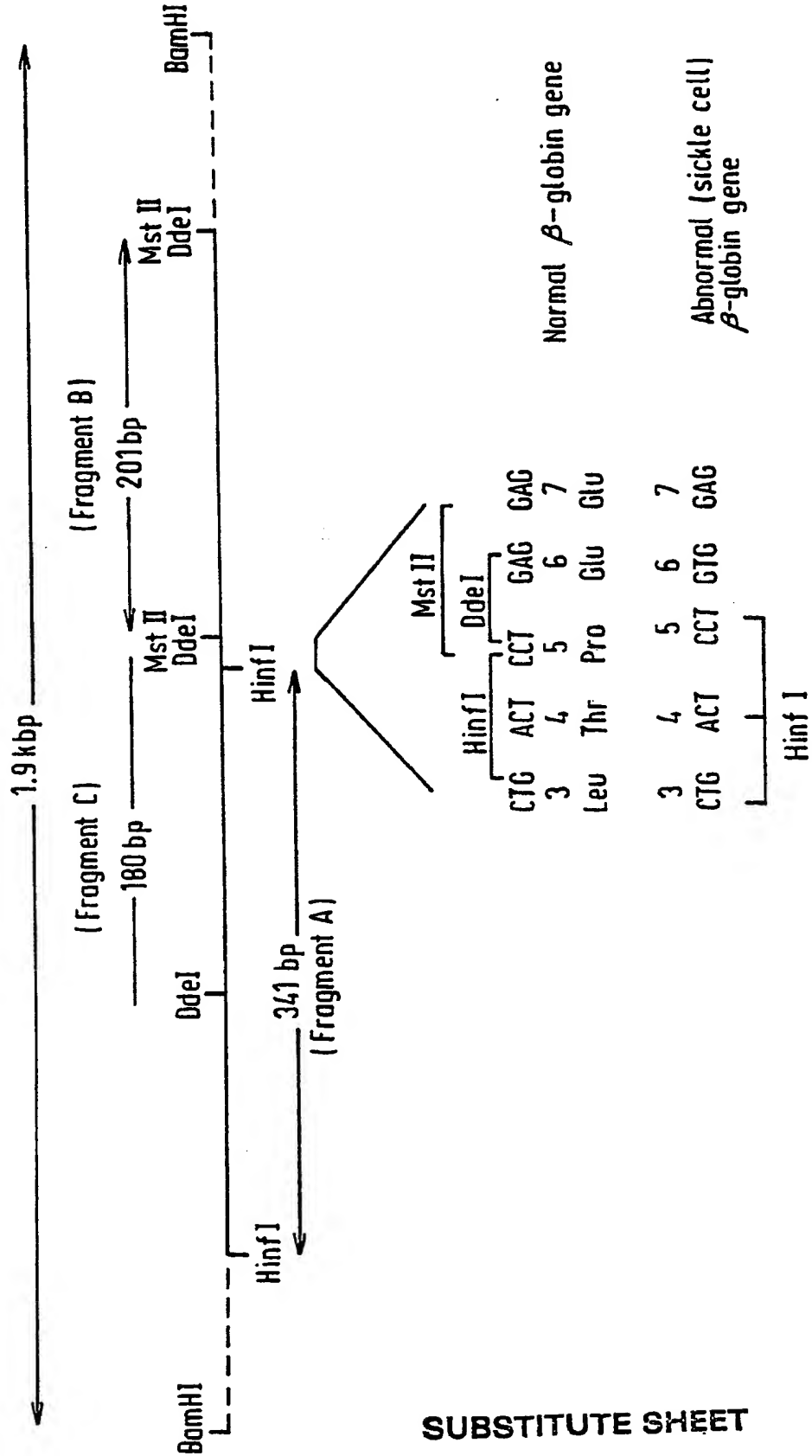
immobilised single-stranded polynucleotide, the said restriction fragment, and said labelled polynucleotide probe, and determining the presence of the marker on the immobilised hybridisation duplex.

- 5 9. A method according to claim 8, wherein the said 180 bp polynucleotide sequence is part of a 341 bp fragment obtained or obtainable by Hinf I digestion of normal human  $\beta$ -globin gene.
- 10 10. A method according to claim 8, wherein the polynucleotide consisting of or containing said 180 bp sequence, as the case may be, and the polynucleotide comprising said 201 bp fragment are obtained by the endonuclease restriction of plasmid  $\beta$ F5.
- 15 11. A method according to claim 10, wherein the immobilised single-stranded polynucleotide is covalently coupled to beads or particles of a cross-linked macroporous cellulosic resin.
- 20 12. A diagnostic kit for the detection of a given nucleotide sequence in a target polynucleotide, comprising as a first component an immobilised polynucleotide reagent comprising solid particles or beads of a cross-linked macroporous resin having a single-stranded polynucleotide sequence complementary to a first section of the nucleotide sequence to be detected covalently bonded to said particles or beads, and as a second component, a polynucleotide probe comprising a single-stranded nucleotide sequence complementary to a second, different section of the nucleotide sequence to be detected, and labelled with a marker.
- 25 13. A diagnostic kit for the detection of the abnormal human (sickle cell)  $\beta$ -globin gene, comprising as a first component an immobilised polynucleotide reagent comprising solid resin particles or beads having covalently bonded thereto a first single-stranded polynucleotide sequence, and as a second component a polynucleotide probe comprising a second single-stranded polynucleotide sequence labelled with a marker, wherein one of said sequences is or comprises said 180 bp polynucleotide sequence obtained or obtainable by
- 30 Dde I digestion of normal human  $\beta$ -globin gene, and the other is or comprises said 201 bp fragment obtained or obtainable by Dde I digestion of normal  $\beta$ -globin gene.
- 35



14. A kit according to claim 13, wherein the said 180 bp polynucleotide sequence is part of a 341 bp fragment obtained or obtainable by Hinf I digestion of normal human  $\beta$ -globin gene.
- 5 15. A kit according to claim 13, wherein the polynucleotide consisting of or containing said 180 bp sequence, as the case may be, and the polynucleotide comprising said 201 bp fragment are obtained by the endonuclease restriction of plasmid  $\beta$ F5.
- 10 16. A kit according to claim 13, wherein said first single-stranded polynucleotide sequence is covalently coupled to particles or beads of a cross-linked macroporous resin.
- 15 17. A kit according to claim 12, wherein the polynucleotide probe is labelled with an enzyme, and the kit also contains, as a third component, means for detecting the enzyme-labelled probe, said means comprising a substrate for said enzyme.
- 20 18. A kit according to claim 13, wherein the polynucleotide probe is labelled with an enzyme, and the kit also contains, as a third component, means for detecting the enzyme-labelled probe, said means comprising a substrate for said enzyme.

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SUBSTITUTE SHEET

# INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 85/00591

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) <sup>6</sup> According to International Patent Classification (IPC) or to both National Classification and IPC IPC <sup>4</sup> : C 12 Q 1/68 // G 01 N 33/546 // G 01 N 33/548																				
<b>II. FIELDS SEARCHED</b> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black; margin: 5px 0;">Minimum Documentation Searched <sup>7</sup></div> <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 30%; border-bottom: 1px solid black;">Classification System</th> <th style="border-bottom: 1px solid black;">Classification Symbols</th> </tr> <tr> <td style="border-right: 1px solid black; padding: 5px;">IPC<sup>4</sup></td> <td style="padding: 5px;">C 12 Q G 01 N</td> </tr> </table> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black; margin: 5px 0;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>8</sup></div>			Classification System	Classification Symbols	IPC <sup>4</sup>	C 12 Q G 01 N														
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<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT <sup>9</sup></b> <table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th style="width: 10%; border-bottom: 1px solid black;">Category <sup>9</sup></th> <th style="border-bottom: 1px solid black;">Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup></th> <th style="width: 10%; border-bottom: 1px solid black;">Relevant to Claim No. <sup>13</sup></th> </tr> </thead> <tbody> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">X</td> <td style="padding: 5px;">EP, A, 0070687 (STANDARD OIL COMPANY) 26 January 1983, see page 4, lines 4-24; page 8, line 29 - page 9, line 32; claims 1,2 (cited in the application) --</td> <td style="text-align: center; vertical-align: top; padding: 5px;">1,2,12</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">P,Y</td> <td style="padding: 5px;">EP, A, 0130523 (MOLECULAR DIAGNOSTICS, INC.) 9 January 1985, see pages 1,2; page 8, line 31 - page 9, line 2; example --</td> <td style="text-align: center; vertical-align: top; padding: 5px;">1,2,5,8-16</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">P,Y</td> <td style="padding: 5px;">EP, A, 0130515 (MOLECULAR DIAGNOSTICS, INC.) 9 January 1985, see the whole document --</td> <td style="text-align: center; vertical-align: top; padding: 5px;">1,2,5,8-16</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">A</td> <td style="padding: 5px;">US, A, 4395486 (L.B. WILSON et al.) 26 July 1983, see the whole document --</td> <td style="text-align: center; vertical-align: top; padding: 5px;">8-10,13-15</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">A</td> <td style="padding: 5px;">Proceedings of the National Academy of Science USA, volume 80, January 1983 B.J. Conner et al.: "Detection of sickle cell <math>\beta^S</math>-globin allele by hybridization with synthetic oligonucleotides", ./.</td> <td style="text-align: center; vertical-align: top; padding: 5px;">./.</td> </tr> </tbody> </table> <div style="font-size: small; margin-top: 10px;"> <div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p><sup>9</sup> Special categories of cited documents: <sup>10</sup></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"A" document member of the same patent family</p> </div> </div> </div>			Category <sup>9</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>	X	EP, A, 0070687 (STANDARD OIL COMPANY) 26 January 1983, see page 4, lines 4-24; page 8, line 29 - page 9, line 32; claims 1,2 (cited in the application) --	1,2,12	P,Y	EP, A, 0130523 (MOLECULAR DIAGNOSTICS, INC.) 9 January 1985, see pages 1,2; page 8, line 31 - page 9, line 2; example --	1,2,5,8-16	P,Y	EP, A, 0130515 (MOLECULAR DIAGNOSTICS, INC.) 9 January 1985, see the whole document --	1,2,5,8-16	A	US, A, 4395486 (L.B. WILSON et al.) 26 July 1983, see the whole document --	8-10,13-15	A	Proceedings of the National Academy of Science USA, volume 80, January 1983 B.J. Conner et al.: "Detection of sickle cell $\beta^S$ -globin allele by hybridization with synthetic oligonucleotides", ./.	./.
Category <sup>9</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>																		
X	EP, A, 0070687 (STANDARD OIL COMPANY) 26 January 1983, see page 4, lines 4-24; page 8, line 29 - page 9, line 32; claims 1,2 (cited in the application) --	1,2,12																		
P,Y	EP, A, 0130523 (MOLECULAR DIAGNOSTICS, INC.) 9 January 1985, see pages 1,2; page 8, line 31 - page 9, line 2; example --	1,2,5,8-16																		
P,Y	EP, A, 0130515 (MOLECULAR DIAGNOSTICS, INC.) 9 January 1985, see the whole document --	1,2,5,8-16																		
A	US, A, 4395486 (L.B. WILSON et al.) 26 July 1983, see the whole document --	8-10,13-15																		
A	Proceedings of the National Academy of Science USA, volume 80, January 1983 B.J. Conner et al.: "Detection of sickle cell $\beta^S$ -globin allele by hybridization with synthetic oligonucleotides", ./.	./.																		
<b>IV. CERTIFICATION</b> <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; border-bottom: 1px solid black; padding: 5px;">Date of the Actual Completion of the International Search</td> <td style="width: 50%; border-bottom: 1px solid black; padding: 5px;">Date of Mailing of this International Search Report</td> </tr> <tr> <td style="border-bottom: 1px solid black; padding: 5px;">2nd April 1986</td> <td style="border-bottom: 1px solid black; padding: 5px;">24 AVR. 1986</td> </tr> <tr> <td style="border-bottom: 1px solid black; padding: 5px;">International Searching Authority</td> <td style="border-bottom: 1px solid black; padding: 5px;">Signature of Authorized Officer</td> </tr> <tr> <td style="padding: 5px;">EUROPEAN PATENT OFFICE</td> <td style="padding: 5px;">M. VAN MOL </td> </tr> </table>			Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	2nd April 1986	24 AVR. 1986	International Searching Authority	Signature of Authorized Officer	EUROPEAN PATENT OFFICE	M. VAN MOL										
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report																			
2nd April 1986	24 AVR. 1986																			
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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
	see pages 278-282 --	8-10,13-15
A	EP, A, 0079139 (ORION CORPORATION LTD.) 18 May 1983, see the whole document (cited in the application) --	1,2,5
A	WO, A, 84/04332 (NATIONAL RESEARCH DEVELOP- MENT CORPORATION) 8 November 1984, see the whole document --	1,2,5,6
A	Chemical Abstracts, volume 96, no. 23, 7 June 1982, Columbus, Ohio, (US) B. Seed: "Diazotizable arylamine cellulose papers for the coupling and hybridization of nucleic acids", see page 356, column 2, abstract no. 196141w, & Nucleic Acids Res. 1982, 1799-1810 (Eng) --	1,2,3
A	EP, A, 0124221 (A.D.B. MALCOLM et al.) 7 November 1984 -----	

# ANNEX TO THE INTERNATIONAL SEARCH REPORT ON

INTERNATIONAL APPLICATION NO. PCT/GB 85/00591 (SA 11720)

This Annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 15/04/86

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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		CA-A- 1180647	08/01/85
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